

CLAIMS

1. Sandwich assay method for detecting the presence of a target molecule in a sample comprising a complex biological fluid, which assay comprises:

- providing a first affinity ligand with affinity for the target molecule, which affinity ligand is capable of being immobilized to a solid support;
- applying the sample in such a way that binding of a target molecule, if present in the sample, to the first affinity ligand is enabled;
- applying a second affinity ligand with affinity for the target molecule, the application enabling binding of the second affinity ligand to the target molecule;
- removing second affinity ligand not bound to target molecule; and
- detecting the presence of the second affinity ligand, such presence being an indicator of the presence of a target molecule in the sample;

the first affinity ligand being immobilized to the solid support at any stage before said detection,

in which method at least one of the first and second affinity ligands is an affinity ligand other than an antibody.

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2. Sandwich assay method according to claim 1, in which the first affinity ligand is provided immobilized to the solid support.

30 3. Sandwich assay method according to claim 1, in which the first affinity ligand is immobilized to the solid support during performance of the method.

4. Sandwich assay method according to any one of claims 2 and 3, in which the solid support is selected from microtiter plates; compact discs comprising micro-

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fluidic channels; protein array chips; membranes; microparticles; pin structures; stick structures; sensor surfaces; and cell surfaces.

5 5. Sandwich assay method according to claim 4, in which the solid support is a microtiter plate.

6. Sandwich assay method according to any one of the preceding claims, which further comprises removing target
10 molecules not bound to the first affinity ligand.

7. Sandwich assay method according to any one of the preceding claims, in which the second affinity ligand is an affinity ligand other than an antibody.

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8. Sandwich assay method according to any one of the preceding claims, in which the first affinity ligand is an affinity ligand other than an antibody.

20 9. Sandwich assay method according to any one of the preceding claims, in which both the first and the second affinity ligand is an affinity ligand other than an antibody.

25 10. Sandwich assay method according to any one of the preceding claims, in which at least one of the first and second affinity ligands is a naturally occurring protein, or a domain thereof.

30 11. Sandwich assay method according to claim 10, in which at least one of the first and second affinity ligands is a naturally occurring bacterial receptor, or a domain thereof.

35 12. Sandwich assay method according to claim 11, in which the naturally occurring bacterial receptor, or domain thereof, is chosen from staphylococcal protein A,

streptococcal protein G and *Peptostreptococcus magnus* protein L, and domains thereof.

13. Sandwich assay method according to any one of
5 the preceding claims, in which at least one of the first and second affinity ligands is a protein which has been selected from a protein library constructed by combinatorial protein engineering.

10 14. Sandwich assay method according to any one of the preceding claims, in which at least one of the first and second affinity ligands is an engineered protein, constructed by using as scaffold a protein domain selected from domains of bacterial receptors; fibronectins;
15 protease inhibitors; retinol binding proteins; bilin binding proteins; amylase inhibitors; CTLA-4; cytochromes; and cellulose binding proteins.

15. Sandwich assay method according to claim 14, in
20 which the scaffold is selected from bacterial receptor domains.

16. Sandwich assay method according to claim 15, in which the scaffold is selected from the immunoglobulin
25 binding domains of staphylococcal protein A.

17. Sandwich assay method according to claim 16, in which the scaffold is the B domain of staphylococcal protein A.

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18. Sandwich assay method according to claim 16, in which the scaffold is the Z domain derived from the B domain of staphylococcal protein A.

35 19. Sandwich assay method according to claim 14, in which the scaffold is selected from the immunoglobulin binding domains of *Peptostreptococcus magnus* protein L.

20. Sandwich assay method according to claim 14, in which the scaffold is selected from the immunoglobulin binding domains of streptococcal protein G.

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21. Sandwich assay method according to claim 14, in which the scaffold is selected from the albumin binding domains of streptococcal protein G.

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22. Sandwich assay method according to any one of claims 14-21, in which the engineered protein used as affinity ligand is selected from a library of variants of the scaffold used.

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23. Sandwich assay method according to claim 22, in which the library is a combinatorial library.

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24. Sandwich assay method according to any one of claims 22-23, in which the library is constructed using phage display technology.

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25. Sandwich assay method according to any one of the preceding claims, in which at least one of the first and second affinity ligands is derived from a library of linear peptides.

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26. Sandwich assay method according to any one of the preceding claims, in which at least one of the first and second affinity ligands is derived from a library of cyclic peptides.

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27. Sandwich assay method according to any one of the preceding claims, in which at least one of the first and second affinity ligands is an oligonucleotide.

28. Sandwich assay method according claim 27, in which the oligonucleotide is an RNA oligonucleotide.

29. Sandwich assay method according to claim 26, in which the oligonucleotide is a DNA oligonucleotide.

5 30. Sandwich assay method according to claim 26, in which the oligonucleotide is an oligonucleotide comprising a mixture of RNA and DNA.

31. Sandwich assay method according to any one of
10 claims 28-30, in which the oligonucleotide is selected from a library of oligonucleotide variants.

32. Sandwich assay method according to any one of the preceding claims, in which the complex biological
15 fluid is selected from serum, plasma, saliva, whole blood, plasma from plasmapheresis, cerebrospinal fluid, amniotic fluid, urine, semen, cord blood, supernatants from cell culture, cell culture media, exsudate and aspirate.

20 33. Sandwich assay method according to any one of the preceding claims, in which the sample is a human sample.

25 34. Sandwich assay method according to claim 33, in which the sample is a human serum sample.

35. Kit for use in a method according to any one of the preceding claims, which kit comprises:
30 - a first affinity ligand with affinity for the target molecule and capable of being immobilized to a solid support;
 - a second affinity ligand with affinity for the target molecule, the presence of which ligand is detect-
35 able;
 - a solid support to which the first affinity ligand is capable of being immobilized,

in which kit at least one of the first and second affinity ligands is an affinity ligand other than an antibody.

5 36. Kit according to claim 35, in which kit the solid support is as defined in any one of claims 4-5.

10 37. Kit according to any one of claims 35-36, in which kit the first and second affinity ligands are as defined in any one of claims 7-31.

15 38. Kit according to any one of claims 35-37, the method in which said kit is used being performed on a sample as defined in any one of claims 32-34.